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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

The overall objective of this proposal is to develop a durable cure for lethal prostate cancer through the elucidation of the role of cancer stem cells in the pathogenesis of the disease. During the past year, we have made the following significant findings: i) CD44, a putative prostate cancer stem cell marker, is localized to neuroendocrine cells in prostate cancer, ii) employing a specific feeder layer, it is possible to model prostate cancer initiation in vitro and iii) classic tissue recombination utilizing primary human prostate cancer cells, to the recapitulate the human disease, does not work. We are currently working to optimize and validate our in vitro model of prostate cancer initiation to facilitate cancer stem cell discovery as well as drug targeting.

**15. SUBJECT TERMS**
Prostate cancer, stem cell, CD44, cancer stem cell

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# Introduction

The overarching goal of this proposal is to develop a durable cure for men with advanced prostate cancer through an improved understanding of the role of human prostate cancer stem cells in the pathogenesis of the disease. To this end, we have proposed the following specific aims:  **1)** to identify and prospectively isolate prostate cancer stem cells from human prostate cancer tissue, **2)** to examine human prostate cancer cell lines, both primary and established, for cells that express cancer stem cell surface markers and the ability to determine therapy resistance *in vitro*, and **3)** to develop an *in vivo* model to assess human prostate cancer stem cell targeted therapy.  The elucidation of the differential biology of cancer stem cells, versus the bulk population of cancer cells, has the potential to lead to the identification of novel therapeutic targets that aim to cripple the driving force behind lethal prostate cancer.

<div align="center">**Body**</div>

***Task 1a:*** Identification of prostate cancer stem cells from human prostate cancer tissue. (months 1-12)

**Aim 1a: Identification of prostate cancer stem cells from human prostate cancer tissue.**

Before pursuing in vivo modeling with sorted cells with markers such as CD44 we pursued establishing the technique without cell sorting (i.e., positive control).  Over the past year we have performed <u>485</u> individual tissue recombination experiments from 17 different prostate cancer specimens obtained at radical prostatectomy. As discussed in our proposal, tissue recombination (TR) was performed by combining single cell suspensions made from areas of suspected prostate cancer with rodent seminal vesicle mesenchyme. Only 2 of 485 tissue recombinants displayed prostate tissue at 3 months. One showed benign human prostate and the other rodent prostate. See figure 1 below.  Notably, our negative findings were corroborated by the Tang lab (collaborator) at MD Anderson. They performed a similar number of TR experiments and were unable to generate a single TR composed of human prostate cancer glands.
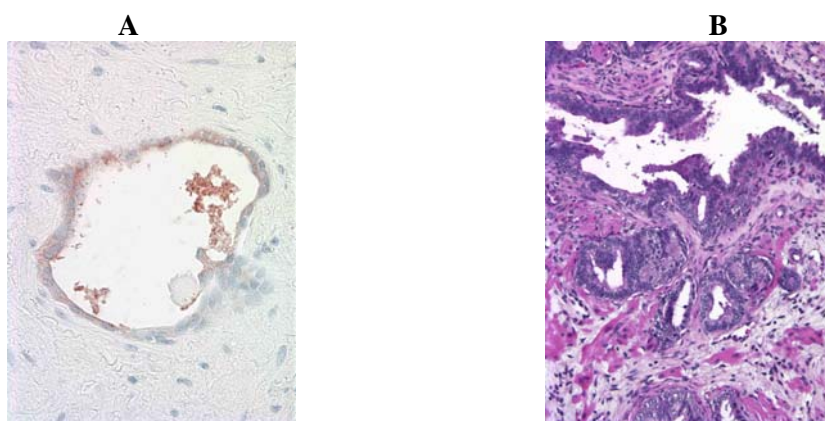
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**Figure 1.**  Tissue recombination with primary unsorted human prostate cancer cells.  Of the almost 500 TRs constructed, only 2 gave evidence of prostate type tissue at harvest.  **A** shows benign human prostate tissue (brown stain for PSA) and **B** displays rodent prostate tissue.

Reasons for our negative findings include: i) starting tissue was not actually prostate cancer but benign or mostly stroma, ii) collagenase/trypsin treatment was too harsh on human cells, or less likely, iii) our technique of TR was sub-optimal (we were able to generate rodent prostate when rodent UGE was combined with rSVM).  At the moment, following discussions with my mentors and collaborators we have halted further TR experiments as described in this aim.

***Task 1b:*** Prospective isolation of prostate cancer stem cells from human prostate cancer tissue. (months 13-30)

**Aim 1b: Prospective isolation of prostate cancer stem cells from human prostate cancer tissue.**

Given the above findings, no work on this sub-aim has been performed to date.

***Task 2a:*** *In vitro* examination of human prostate cancer cell lines, both primary and established, for cells that express cancer stem cell surface markers. (months 6-24)

**Aim 2a: *In vitro* examination of human prostate cancer cell lines, both primary and established, for cells that express cancer stem cell surface markers.**

Based upon conversations with mentor Craig T. Jordan and collaborator Dean Tang, we have focused initially on CD44.  We have completed a detailed analysis of CD44 expression in three common human prostate

<div align="right">5</div>

cancer cell lines and in human prostate cancer tissue. This work has been published and the DoD cited as a funding source. See reference below (reprint attached to appendix).

**Palapattu GS**, Wu G, Silvers C, Martin, HB, Williams K, Salamone L, Bushnell T, Huang LS, Yang Q, Huang J. Selective expression of CD44, a putative prostate cancer stem cell marker, in neuroendocrine tumor cells of human prostate cancer. *Prostate*, 15;69(7): 787-98, 2009.

We have encountered significant problems with culturing and maintaining primary human prostate cancer cells *in vitro*. Per discussions with others in the field, this is not altogether unexpected. To overcome this problem we have begun studies using rSVM as a feeder layer. This idea borrows from Cuhna's[1] work on *in vivo* TR and work published by Witte[2] and Isaacs[3] on the use of feeder layers in prostate cell culture. In essence, we are creating 'TR in a dish'. Initial studies with this technique on the non-adherent cell line LAPC-9 and 2 other xenograft maintained human prostate cancer cell lines have shown promise. We have termed the 3-D structures formed by single cell suspensions upon this feeder layer 'glandoids' (see figure 2). Glandoids typically form with 21-27 days after plating on rSVM that has been irradiated to 30Gy to prevent overgrowth, and are composed of all cell types relevant in human prostate cancer (luminal cells, NE cells; no basal cells).
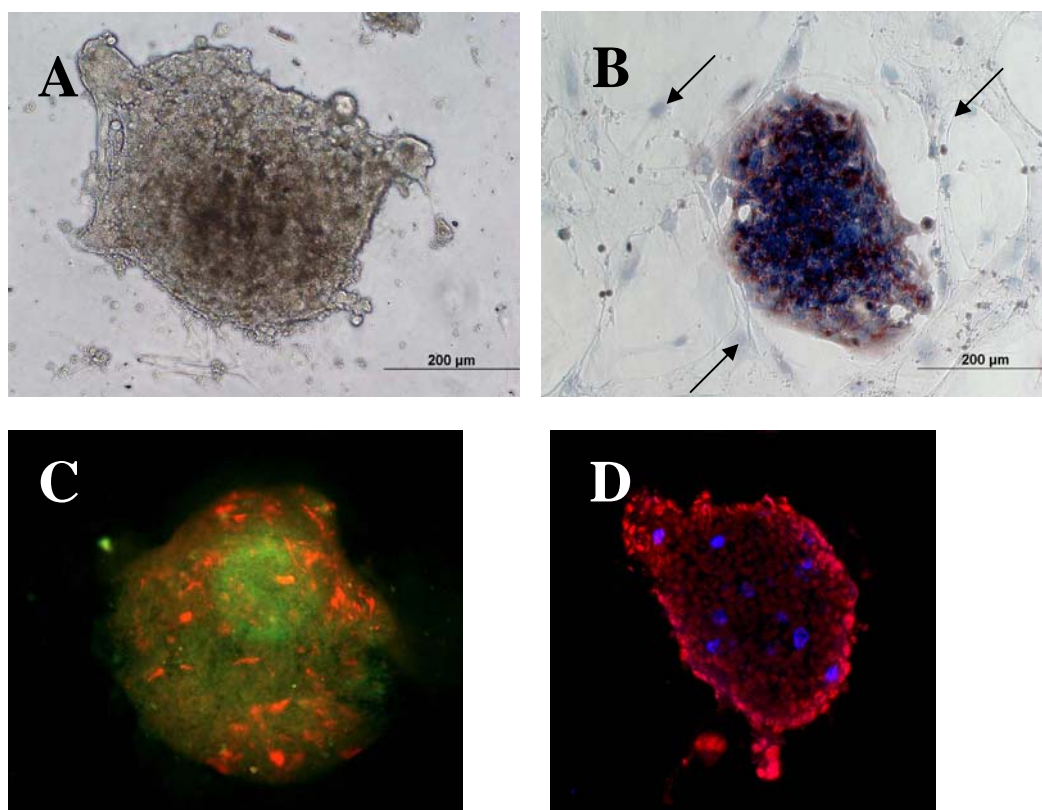


**Figure 2.** *In vitro* 'glandoids'. **A** represents light microscopy of a glandoid at 21 days. **B** shows a glandoid upon rSVM (arrows). Brown (DAB) stain is for PSA; blue nuclear stain. **C** shows immunofluorescent analysis of a glandoid for PSA (green) and cytokeratin 8 (orange). Panel **D** demonstrates rare neuroendocrine cells in glandoids (blue=chromogranin A; red=cytokeratin 8).

Interestingly, we have also shown that primary glandoids can give rise to daughter (secondary) glandoids- implying this assay may also be able test self-renewal (an important attribute of stem cells). We are currently evaluating the tumorigenicity of these glandoids and testing the assay for clonogenicity and reproducibility with other human prostate cancer cell lines. We are also studying the composition and tumorigenicty of secondary glandoids. Our goal is to develop this assay as a valid model of human prostate cancer initiation with either primary human prostate cancer cells and/or non-adherent prostate cancer xenograft cell lines. The successful development of this assay will allow us to assess/screen candidate compounds *in vitro* for their impact on tumor initiation/stem cell activity.

***Task 2b:*** Assessment of the ability of human prostate cancer cells that possess cancer stem cell surface antigen expression to determine therapy resistance *in vitro*. (months 14-28)

**Aim 2b: Assessment of the ability of human prostate cancer cells that possess cancer stem cell surface antigen expression to determine therapy resistance *in vitro*.**

No work on this sub-aim has been performed to date.

***Task 3:*** Development of an *in vivo* model to assess human prostate cancer stem cell targeted therapy. (months 30-60)

**Aim 3:  Development of an *in vivo* model to assess human prostate cancer stem cell targeted therapy.**

No work on this aim has been performed to date.

**Key Research and Training Accomplishments**

Research accomplishments:
- Characterization of the putative cancer stem cell marker CD44 in human prostate cancer tissues
- Development of a novel in vitro assay of prostate cancer initiation
- Definitively invalidating current methods of tissue recombination as a viable method of reconstituting the human disease in vivo.

Training accomplishments:
- Coursework:
  - Enroll in Cancer Biology, Cell Biology and Molecular Biology/Genetics courses; 1/year: *pending*
  - Continue as Instructor/Faculty of medical school/graduate courses: completed as stated in application

- Conferences/journal clubs:
  - Attend cancer stem cell seminar; monthly: completed
  - Meet with mentors (Drs. Messing and Jordan) to discuss research progress/career development: completed
  - Continue leadership/active roles in Urology Grand Rounds, GU tumor board, Urology journal club, Wilmot Cancer Center Research seminar, Urology PI Research seminar: all completed

- Clinical responsibilities
  - Continue Urology clinic and GU Multi-disciplinary clinic: completed
  - Continue operative schedule: completed

Professional accomplishment: recruited/promoted to chief of urologic oncology at The Methodist Hospital in Houston, Texas with protected salary and 50% protected research time. DoD grant transfer initiated; Dr. Carolyn Best at the DoD is aware. I fully intend to continue my research on prostate cancer stem cells and fulfill the obligations of this DoD award. Newly identified mentors: Malcolm Brenner, MD, PhD and Christopher Logothetis, MD.

**Reportable Outcomes**

1. Manuscript:

   **Palapattu GS**, Wu G, Silvers C, Martin, HB, Williams K, Salamone L, Bushnell T, Huang LS, Yang Q, Huang J. Selective expression of CD44, a putative prostate cancer stem cell marker, in neuroendocrine tumor cells of human prostate cancer. *Prostate*, 15;69(7): 787-98, 2009.

2. Employment opportunity:

   Professional: recruited/promoted to chief of urologic oncology at The Methodist Hospital in Houston, Texas with protected salary and 50% protected research time. My hire was facilitated, in part, by my DoD award. A central research theme at The Methodist Hospital is cancer stem cell biology.

No other reportable outcomes to report.

**Conclusions**

From the studies we have completed to date we conclude that: i) the putative cancer stem cell marker CD44 is selectively expressed in neuroendocrine cells in human prostate cancer, ii) *in vivo* modeling of human prostate cancer with single cells obtained from primary patient samples via tissue recombination is extremely challenging and iii) *in vitro* modeling of human prostate cancer initiation is possible.

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# Selective Expression of CD44, a Putative Prostate Cancer Stem Cell Marker, in NeuroendocrineTumor Cells of Human Prostate Cancer

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Los Angeles, California*

**BACKGROUND.** Hormonal therapy is effective for advanced prostate cancer (PC) but the disease often recurs and becomes hormone-refractory. It is hypothesized that a subpopulation of cancer cells, that is, cancer stem cells (CSCs), survives hormonal therapy and leads to tumor recurrence. CD44 expression was shown to identify tumor cells with CSC features. PC contains secretory type epithelial cells and a minor population of neuroendocrine cells. Neuroendocrine cells do not express androgen receptor and are quiescent, features associated with CSCs. The purpose of the study was to determine the expression of CD44 in human PC and its relationship to neuroendocrine tumor cells.
**METHODS.** Immunohistochemistry and immunofluorescence were performed to study CD44 expression in PC cell lines, single cells from fresh PC tissue and archival tissue sections of PC. We then determined if CD44 + cells represent neuroendocrine tumor cells.
**RESULTS.** In human PC cell lines, expression of CD44 is associated with cells of NE phenotype. In human PC tissues, NE tumor cells are virtually all positive for CD44 and CD44 + cells, excluding lymphocytes, are all NE tumor cells.
**CONCLUSIONS.** Selective expression of the stem cell-associated marker CD44 in NE tumor cells of PC, in combination with their other known features, further supports the significance of such cells in therapy resistance and tumor recurrence. *Prostate 69: 787–798, 2009.*
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KEY WORDS: prostate cancer; neuroendocrine cell; CD44; cancer stem cell

## INTRODUCTION

Prostate cancer (PC) is the most commonly diagnosed cancer and the second leading cause of cancer-related mortality [1]. Multiple options exist for the treatment of organ-confined PC. The primary treatment of choice for advanced/metastatic PC, however, is hormonal therapy [2], consisting of androgen ablation and/or inhibition of androgen action with anti-androgens. Although most patients initially respond to this therapy, the tumor commonly recurs and enters an

androgen-independent (hormone-refractory) stage for which no durable effective therapy is currently available.

Cancer cells within a given tumor were once considered homogeneous, a situation wherein each cell would have equal malignant potential. Data over the past decade, however, have challenged this hypothesis and established that a hierarchy often exists among tumor cells within a given cancer [3]. In vitro and in vivo assays in hematopoietic cancers as well as breast, brain and colon cancer have shown that only a minor subpopulation (typically 1–2%) of tumor cells possesses the ability to self-renew and recreate the entire tumor, inclusive of all cell types [4]. Such "tumor initiating" cells are termed cancer stem cells (CSCs) [5].

Unlike the bulk cancer cells, CSCs do not express differentiation markers and are typically quiescent. As a result, they may be resistant to traditional therapies that depend on continuous cell cycle activity, such as chemotherapy and radiation. The CSC model predicts that potential CSCs within PC are quiescent and do not express the luminal differentiation markers androgen receptor (AR) and prostate specific antigen (PSA) [6–8]. Therefore, these cells are likely androgen-independent and should survive androgen ablation therapy, leading to tumor recurrence [9]. To date, the critical experiment demonstrating the identification of prostate CSCs from primary human tissue with subsequent illustration that the proffered CSC is tumor-initiating in vivo has not been reported. Nonetheless, many groups have reported potential markers that may be associated with prostate CSCs, including the cell surface markers CD44, integrin α2β1, CD133, CXCR4 and breast cancer resistance protein (BCRP) [10–16] as well as cytokeratin 5/18 double positive intermediate cells [17,18] and the side population of cells [19].

In a comprehensive in vitro and in vivo study using cell lines and xenograft tumor models, Patrawala et al. [20] provided compelling evidence that CD44 expression is associated with stem/progenitor cells of PC. They found a general correlation between the proportion of CD44+ cells and tumorigenicity in PC cell lines, with the highly aggressive, androgen-independent PC3 cells and DU145 cells containing more CD44+ cells than the less aggressive, androgen-dependent LNCaP cells. CD44+ cells had higher clonogenicity and tumorigenicity and also expressed higher levels of stem cell-associated genes than CD44− cells. In addition, the authors noted that CD44+ cells did not express AR, while AR was exclusively detected in the CD44− cell population. Importantly, CD44+, AR−, PC cells were capable of generating CD44−, AR+ tumor cells in in vitro and in vivo assays [20]. These results have provided strong evidence that CD44 is associated with stem/progenitor cells in PC. Interestingly, in a landmark report, Leong et al. [21] showed that a single cell expressing CD44 as well as a few other stem cell markers can be used to generate mouse prostate. Expression of CD44, however, has not been studied in detail in human PC tissue. If CD44 expression is associated with human prostate CSCs, one might expect that CD44+ tumor cells would be scattered among the more abundant bulk tumor cells that possess features of luminal differentiation including expression of AR and PSA.

It is well established that PC is histologically heterogeneous. The majority of malignant cells are of the secretory type epithelial cells that express AR and secrete PSA. Notably, every case of PC also contains a minor population of cells that have neuron-like morphology and produce biogenic amines and neuropeptides. These cells have been termed neuroendocrine (NE) cells and they reside in the basal layer in benign prostate acini. We and others have characterized these NE cells in PC and shown that unlike the bulk secretory type tumor cells, the NE tumor cells are quiescent and do not express AR or PSA [22–24]. Several groups, including our own, have proposed that these NE cells may be resistant to hormonal therapy and therefore responsible for tumor recurrence following androgen ablation (reviewed in Refs. [25–27]). Here, we report our results showing that the putative CSC marker CD44 is selectively expressed in NE tumor cells of PC, further supporting the importance of such cells in therapy resistance and tumor recurrence and raising interesting questions about the relationship of the NE tumor cells to the elusive PC stem cell.

## MATERIALS AND METHODS

### Established Cell Lines

PC-3 (CRL-1435), DU145 (HTB-81), and LNCaP (CRL-1740) cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). All cell lines were routinely maintained in RPMI 1640 (Invitrogen Corp., Carlsbad, CA) containing Penicillin-Streptomycin (Invitrogen Corp.) and 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA).

### Fresh Human Surgical Samples

Fresh human prostate tissue was obtained from patients undergoing radical prostatectomy, in accordance with the protocol approved by the University of Rochester Research Subjects Review Board. Upon removal, fresh prostate tissue was cut into 1 mm cubes using sterile disposable scalpels. After washing in RPMI the tissue was incubated in a $CO_2$ tissue culture incubator overnight in 112 U/ml hyaluronidase (Sigma

H-3506) and 250 U/ml collagenase 1 (Worthington Biochemical MIE4816). The resultant single cell suspension was neutralized by repeated washing in RPMI/5% FBS followed by resuspension in FACS buffer (1% FBS in D-PBS [Invitrogen Corp.], 0.01% DNase, Sigma, St. Louis, MO). All samples were filtered through a 100 µm cell strainer prior to staining.

## Tissue Microarray: Immunohistochemistry and Immunofluorescence

The prostate TMA was constructed as previously described [28]. Briefly, 73 prostatectomy specimens were reviewed and areas containing prostate adenocarcinoma were marked for sampling. Tumors ranged from Gleason patterns 2 to 5. Two to three cores per samples, measuring 0.6 mm in diameter, were obtained from selected regions in each donor paraffin block and transferred to a recipient paraffin block and the resulting block contained a total of 200 cores. A section was obtained from the TMA for H&E staining as quality control and unstained sections were used for immunohistochemical and immunofluorescence staining.

The procedure for immunohistochemical staining has been described in detail previously [28]. The TMA sections were stained with a mouse monoclonal antibody against chromogranin A (Chemicon International, Inc., Temecula, CA, Clone 2H10, used at 1:1,000), and a rat monoclonal antibody against CD44 (eBioscience, San Diego, CA., Clone IM7, used at 1:1,000). Paraffin embedded tissues were sectioned at 5 µm thickness and antigen retrieval was performed with pre-heated (95–99°C) Citrate Buffer, pH 6.1 (DakoCytomation, Carpinteria, CA) in a Black and Decker steamer (Shelton, CT, Model HS800) for 30 min. The sections were incubated with the primary antibodies at room temperature for 60 min (CD44) or 45 min (chromogranin A), followed by incubation for 30 min with the link antibody (rabbit or mouse) - labeled polymer-HRP (Envision Plus System, DakoCytomation). Slides were developed with AEC + (DakoCytomation) and counterstained in Modified Mayers Hematoxylin.

For immunofluorescence staining of the TMA section, Antigen retrieval was performed as described above. Anti-CD44 (same source as above, used at 1:200), anti-CD45 (Dako North America, Inc., Carpinteria, CA; M0701, 1:100), and anti-chromogranin A (Dako; A0430, 1:1,000) antibodies were incubated with the TMA slide overnight at room temperature. The slide was then incubated with secondary antibodies (goat anti-rat IgG FITC [Invitrogen Corp.; 62-9511, 1:200], Alexa Fluor 546 goat anti-mouse [Invitrogen Corp.; A-11003, 1:200], and Alexa Fluor 633 F(ab)$_2$ fragment

of goat anti-rabbit [Invitrogen Corp.; A-21072, 1:200]) for 40 min at room temperature. The slide was mounted with a coverslip using Vectashield HardSet Mounting Medium with DAPI (Vector; H-1500). Tissue cores were photographed individually with a Leica TCS SP Spectral Confocal microscope. Subsequently, the coverslip was removed and the TMA stained with H&E. The H&E-stained tissue cores were then photographed with a Leica DM5000 B microscope. Cancerous areas in each core were marked by a pathologist (JH) and the nuclei manually marked in each digital image and counted using the particle analysis feature of NIH ImageJ software (http://rsb.info.nih.gov/ij/). Marked cells in cancerous regions were examined for fluorescence in the corresponding confocal images, and the number of positive cells recorded.

## Quantitative RT-PCR

Detailed method has been described previously [29]. Total RNA was isolated from cells with RNeasy® Kit (Qiagen) according to the manufacturers instructions. RNA was reverse transcribed by Transcriptor reverse transcriptase (Roche, Germany) with random hexamers (Promega). The following specific forward and reverse primers were used: for NSE, 5′-AGCTGC CCCTGCCTTAC-3′ and 5′-GAGACAAACAGCGTTA CTTAG-3′; for chromogranin A, 5′-GCGGTGGAAG AGCCATCAT-3′ and 5′-TCTGTGGCTTCACCACTT TTCTC-3′; for β-actin, 5′-GCGGGAAATCGTGCGT GACATT-3′ and 5′-GATGGAGTTGAAGGTAGTTTC GTG-3′.

Real time PCR was performed with iQ™ SYBR® Green Supermix in an iCycler iQ System (Bio-Rad) using the SYBR Green Detection protocol. Total reaction volume was 20 µl and a cycle consists of 95°C for 5 min, 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, for a total of 45 cycles followed by 72°C for 5 min.

## Western Blotting

Detailed method has been described previously [29]. Briefly, cells were washed twice with cold PBS and lysed in RIPA lysis buffer for 30 min on ice. The cells were sheared twice through a 20 gauge needle and centrifuged at 14,000 rpm for 15 min at 4°C. The protein concentration in the supernatant was determined with the Bio-Rad Protein Assay kit. Equal amounts of protein were separated on 10% SDS–PAGE gels, transferred to nitrocellulose membrane with Semi-Dry Transfer Cell (Bio-Rad). The membrane was blocked with TBS containing 5% w/v nonfat dry milk, and hybridized with primary antibody in 2%w/v nonfat dry milk, followed by incubation with secondary antibody and detected with an ECL kit (BioRad).

## Flow Cytometry

To minimize non-specific binding, single cells suspensions were treated with FC block before staining with PE-Cy5 labeled anti-human CD44 antibody for 20 min on ice. After washing with PBS containing 0.5% BSA, the cells were resuspended in the same solution and DAPI was added to a final concentration of 1 µg/ml. All flow-cytometry studies were performed using either a Becton Dickinson FACSAria or LSRII flow cytometer. For sorting experiments, the cells were maintained at 4°C during the sort, and an 85 µm nozzle was used. Cells were sorted into RPMI medium. Populations were analyzed post-sort to ensure purity of sorts before progressing with additional experiments. For cells that did not have a clear positive and negative distribution, the top 10% and bottom 10% of cells were sorted and designated as CD44 high and CD44 low.

## Cytospin: Immunofluorescence Analysis

Cytospin preparations of PC cells were fixed in methanol for 10 min at −20°C, rehydrated in PBS (Sigma-Aldrich Corp., St. Louis, MO; D5773), and blocked in 5% normal goat serum (Rockland Immunochemicals, Inc., Gilbertsville, PA; B304) for 30 min. The slides were incubated with antibodies against CD44 (as above, used at 1:200) and neuron-specific enolase (NSE; Dako North America, Inc.; M0873, 1:50) overnight at 4°C followed by incubation with secondary antibodies (goat anti-rat IgG FITC and Alexa Fluor 546 goat anti-mouse, as described above) for 40 min at room temperature. For cell lines, the slides were mounted with coverslips using Vectashield HardSet Mounting Medium with DAPI. For cells derived from fresh prostatecomy specimens, the slides were stained with Hoechst 33258 (Sigma-Aldrich Corp.; 861405) for 10 min prior to coversliping. Fluorescence micrographs were obtained with a Leica DM5000 B microscope. Cellular co-expression of CD44 and NSE was quantified in fluorescence micrographs of PC3 and DU145 cytospin preparations. Total cell number was derived by counting nuclei in the DAPI images using the particle analysis feature of NIH ImageJ software (http://rsb.info.nih.gov/ij/). Cell masks were generated in ImageJ using a composite of the CD44 and NSE fluorescence signals; the masks were used to derive the mean pixel value of each fluorescence signal within individual cells.

## Statistical Analysis

The analysis included calculation of the Pearson correlations and non-parametric Spearmans correlations between CD44 and NSE levels. Linear regression analysis was also implemented with an assessment of residuals as a check on the assumptions of normally distributed errors with constant variance. If the assumptions seemed to be violated, log-transformed values were used to produce more normally distributed residuals. Statistical outliers were defined as the standardized residuals values >3 or ≤3. Then the models were rerun without the outliers and the results with and without outliers were compared.

## RESULTS

### Expression of CD44 and NE markers in Human PC Cell Lines

Flow cytometric studies demonstrated that among the three well-characterized PC cell lines (LNCaP, DU145, and PC-3), PC3 cells were nearly 100% positive for CD44 expression, and ~60% of DU145 cells were positive for CD44. LNCaP cells were nearly entirely negative for CD44 (Fig. 1A). These results are consistent with the findings reported by Patrawala et al. [20] We then studied if CD44 expression correlates with NE phenotype in these cell lines. The most commonly used NE markers include chromogranin A and NSE [25]. As shown in Figure 1B,C, the largely CD44− LNCaP cell line did not express NE markers, while NE marker mRNA was detected, in varying degrees, in the CD44 + DU145 cells and PC3 cells. The observed expression pattern of chromogranin A and NSE mRNAs paralleled that of CD44 expression among the three cell lines (i.e., PC3 had the highest CD44 content and the highest NE marker mRNA concentration).

To further characterize the association of CD44 expression with NE markers, we used fluorescence activated cell sorting (FACS) to sort LNCaP, PC3 and DU145 cells into CD44 high and CD44 low expressing subpopulations. As shown in Figure 2A,B, within each cell line studied, NE marker expression was enriched in the CD44 high population versus unsorted and CD44 low cells. This finding was confirmed with Western blot analysis as depicted in Figure 2C.

We next examined the expression of CD44 and the NE marker NSE in the three cell lines by immunofluorescence after the cells were spun onto glass slides by the cytospin technique. The advantage of this technique is that the expression of multiple proteins can be simultaneously studied in the same cells. As shown in Figure 3A, LNCaP cells were essentially negative for both CD44 and NSE and PC3 cells were nearly all positive for both CD44 and NSE. DU145 cells displayed a wide range of staining, from totally negative to brightly positive for both CD44 and NSE. In general, CD44 negative DU145 cells were negative for NSE while CD44 positive DU145 cells were positive for NSE.
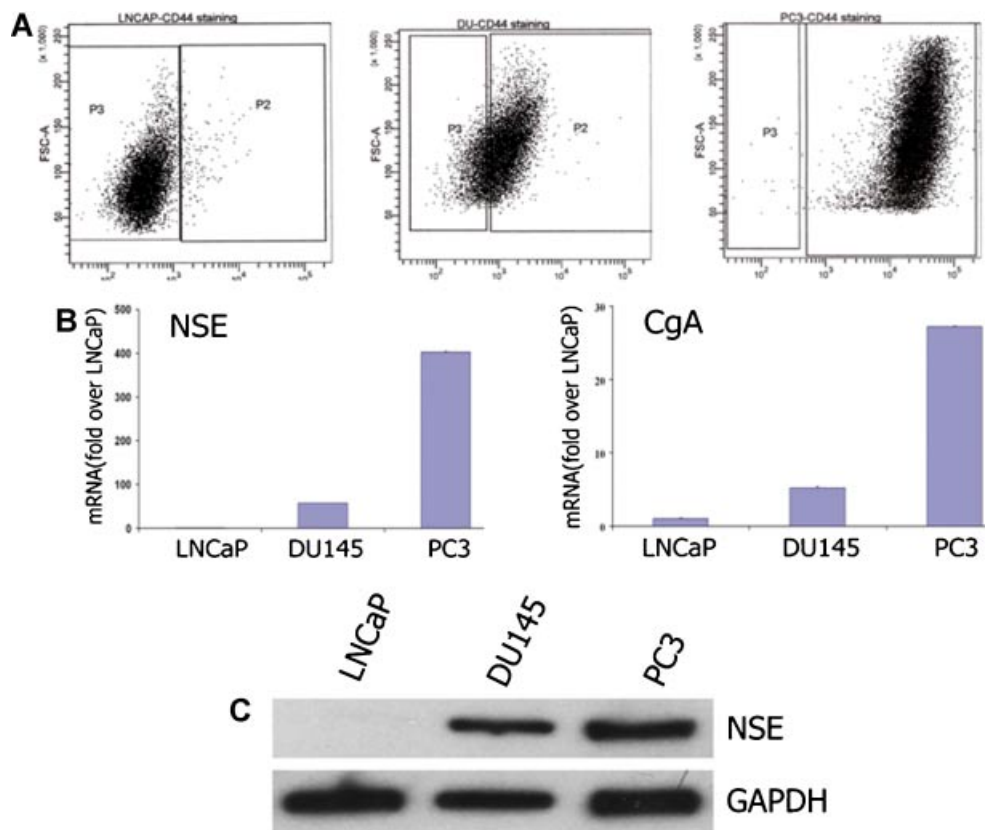
**Fig. I.** Expression of CD44 and NE cell marker in human prostate cancer cell lines. **A**: Flow cytometry examining CD44 expression in LNCaP, DUI45, and PC3 cells. LNCaP cells are mostly negative for CD44. Approximately 60% of DUI45 cells are positive for CD44 while PC3 cells are mostly positive for CD44. **B**: RT-PCR analysis of the mRNA levels of NE cell markers in PC cell lines. Expression of NE cell markers neuron specific enolase (NSE) and chromogranin A (CgA) mirrored CD44 expression with LNCaP cells expressing lowest levels while PC3 cells expressed higher levels. **C**: Western blot analysis of the protein levels of NSE in PC cell lines. Similarly, NSE protein was undetectable in LNCaP cells and highest in PC3 cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Statistical analysis was performed to study the correlation between CD44 and NSE expression after the image intensity of individual cells was captured, as described in Materials and Methods Section. The correlations between CD44 and NSE were 0.6901 in DU145 cells and 0.6518 in PC3 cells. The correlations based on log-transformed values were similar, 0.6860 and 0.6585 respectively. The non-parametric Spearman correlation was similar for DU145 cells (0.6764), and higher for PC3 cells (0.7516). The linear model for DU145 cells with CD44 as the predictor and NSE as the response had an $R^2$ of 0.4763, and for PC3 cells the $R^2$ was 0.4249. Both models were highly significant ($P < 0.0001$). The models identified 3 and 4 outliers for DU145 and PC3 cells respectively. After removing the outliers, the $R^2$ increased to 0.4944 and 0.5019, respectively. The residual plots showed that the assumption of normal error distribution was satisfactory. Nevertheless, the linear models for log-transformed values were explored and their $R^2$ values were similar to those without transformation, 0.4705 and 0.4336 respectively. Figure 3B shows

the linear fits based on raw values (without log transformation). These data indicate that on cytospin examination, there is a strong correlation between the expression of CD44 and NSE, suggesting that CD44 expression is associated with NE phenotype in such cells.

## Expression of CD44 and NE Markers in Primary Fresh Human PC Cells

To further establish the relationship between CD44 expression and NE markers in PC, we obtained fresh PC tissue from seven prostatectomy specimens immediately upon removal of the prostate. Single cell suspensions were obtained and flow-sorted into CD44 high and CD44 low cells. The small number of cells derived from the surgical specimens allowed only quantitative real-time PCR analysis. In every case, the levels of NE markers were much higher in the CD44 high cells than those in the CD44 low cells and the difference was statistically different in each case (Fig. 4A,B).
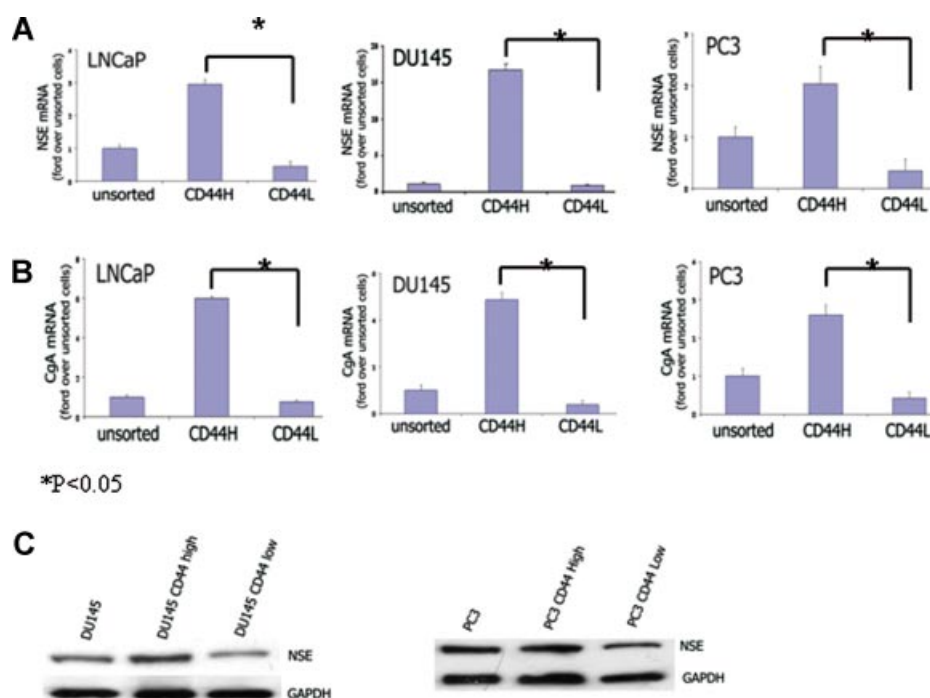
**Fig. 2.** Expression of NE markers in CD44 high and CD44 low fractions of human prostate cancer cell lines. RT-PCR for mRNA levels of NSE (**A**) and CgA (**B**) revealed enrichment for NE marker expression in CD44 high (CD44H) populations versus CD44 low (CD44L) and unsorted populations. This finding was confirmed at the protein level by Western blot for NSE (**C**). The low level of CD44 expression within LNCaP cells precluded accurate sorting and adequate protein extraction from CD44 + LNCaP cells for protein analysis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The single cell suspensions from fresh PC tissue were also spun onto slides by cytospin method and double-stained by immunofluorescence for the expression of CD44 and chromogranin A. As predicted, very few cells were NE cells. Similarly, in these single cell suspensions, CD44 expression was limited to NE tumor cells (Fig. 4C).

### Expression of CD44 in Benign and Malignant Prostate Tissue

We then performed immunohistochemistry to study the expression of CD44 in archival, formalin-fixed and paraffin-embedded sections of human PC. Positive staining was defined as strong membrane staining, consistent with CD44 being a cell surface protein. In benign prostate tissue, all basal cells expressed CD44, consistent with previous reports [30–32] (Fig. 5A). Lymphocytes and nerves were also positive for CD44 (Fig. 5B,C). PC is characterized by the absence of basal cells and the proliferation of luminal type malignant epithelial cells. Although the majority of cancer cells were negative for CD44, there were scattered individual cells or small nests of cells that displayed CD44 expression with a distinct membranous staining pattern. The distribution of the CD44 + cells was reminiscent of NE tumor cells of PC (Fig. 5D).

### Co-Expression of CD44 and Chromogranin A in Human PC Tissue

We next performed experiments to confirm that CD44 + cells in PC tissues are in fact NE cells. We prepared adjacent sections of human PC tissue (5 µm apart) which contained virtually identical tumor cells. The first section was stained with an anti-CD44 antibody and the second section stained with an anti-chromogranin A antibody to highlight NE cells. Chromogranin A positive NE cells displayed cytoplasmic staining and were scattered among the more abundant cancerous epithelial cells. In the adjacent section, CD44 + cells demonstrated a membrane staining pattern and similarly appeared as single cells and small nests of cells surrounded by more abundant CD44− cells. When the same microscopic fields from the two adjacent sections were compared, cells that were positive for CD44 were also noted to be positive for chromogranin A and vice versa (for illustration, an area with abundant NE cells are shown in Fig. 6A).

In order to definitively prove the relationship of CD44 expression with NE cells in PC, we employed an immunofluorescence method so that multiple antibodies could be used to stain the same tumor cells. Our pilot studies indicated that NE cells within tumors were all positive for CD44 but CD44 positive cells were
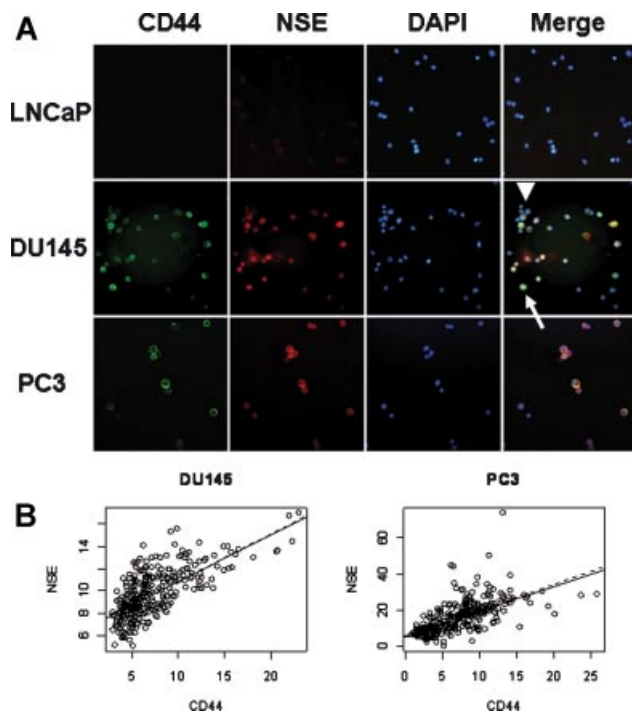
**Fig. 3.** Co-expression of NSE and CD44 in human prostate cancer cell lines. **A**: Immunofluorescence studies on cytospin samples with antibodies against CD44, NSE (with DAPI staining nuclei) show co-expression of CD44 and NSE in the same cells. LNCaP cells are double negative for the two markers and PC3 cells are double positive. The majority of DUI45 cells are double positive (arrow) but a minority are double negative (arrowhead) (magnification 400×). **B**: Linear fits of CD44 and NSE for DUI45 and PC3 cells. The linear model with CD44 as the predictor and NSE as the response for DUI45 cells yields an $R^2$ of 0.4763 and for PC3 cells 0.4249. Both models are highly significant ($P < 0.0001$). The dash line is the fit with outliers and the solid line without outliers. These data indicate that on cytospin examination, CD44 and NSE expressions were closely associated with each other in individual cells. [Color figure can be viewed in the online issue, which is available at www.interscience. wiley.com.]



**Fig. 4.** Association of CD44 expression with NE cells in fresh primary human prostate cancer cells. **A**: Quantitative RT-PCR analysis performed on sorted single cell suspensions obtained from seven cases of fresh radical prostatectomy specimens revealed that NE markers CgA and NSE expression was significantly higher in the CD44 high versus the CD44 low population. **B**: Single cell suspension obtained from a case of fresh radical prostatectomy specimen was co-stained by immunofluorescence for the expression of CD44 and CgA (nuclei stained by Hoechst 33258). A single NE cell is the only CD44+ cell (long arrow). The other bright spot (short arrow) in the field is a contaminant as it is not associated with a nucleus (magnification 400×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

composed of NE tumor cells and lymphocytes that commonly infiltrate PC. Therefore, we co-stained a section of a tissue microarray that contained 200 cores of PC tissue from 73 different radical prostatectomy cases for the expression of CD44, chromogranin A and CD45 (a marker of leukocytes including lymphocytes). The areas of cancer in each core were marked and the number of nuclei (stained by DAPI, including cancer cells + lymphocytes) in cancerous areas of each core counted manually, which ranged from 40 to 1,755 per core with a total of 61,070 cells surveyed in aggregate. Among them, 147 cells were positive for chromogranin A (NE cells) comprising 0.2% of all nuclei. Of these, 132 (89.8%) were CD44+. Lymphocytes (CD45+) comprised 0.8% (516 cells) of all nuclei (Table I). Approximately 10% (15 cells) of NE cells were negative for both CD44 and CD45. Among the 648 CD44+ cells
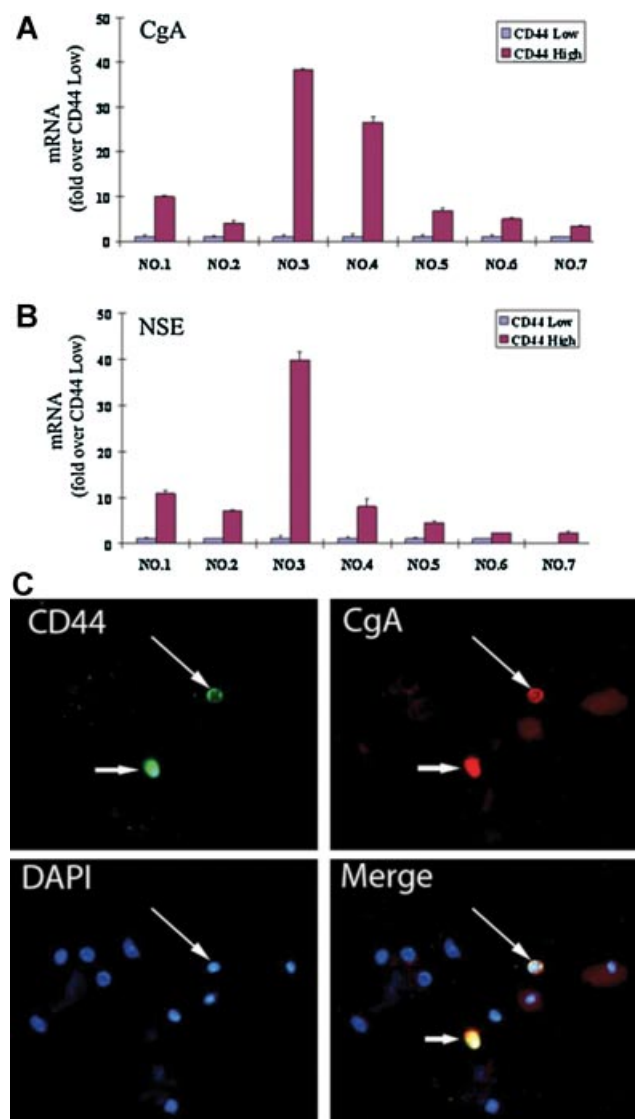
counted, 132 (20.4%) were positive for chromogranin A, 516 (79.6%) were positive for CD45 and 2 (0.3%) were positive for both chromogranin A and CD45 (faint) (Table I). Of the 61,070 cells reviewed, 2 were faintly triple positive for CD44, CD45 and CgA. These two cells were not included in the above analysis. Therefore, with
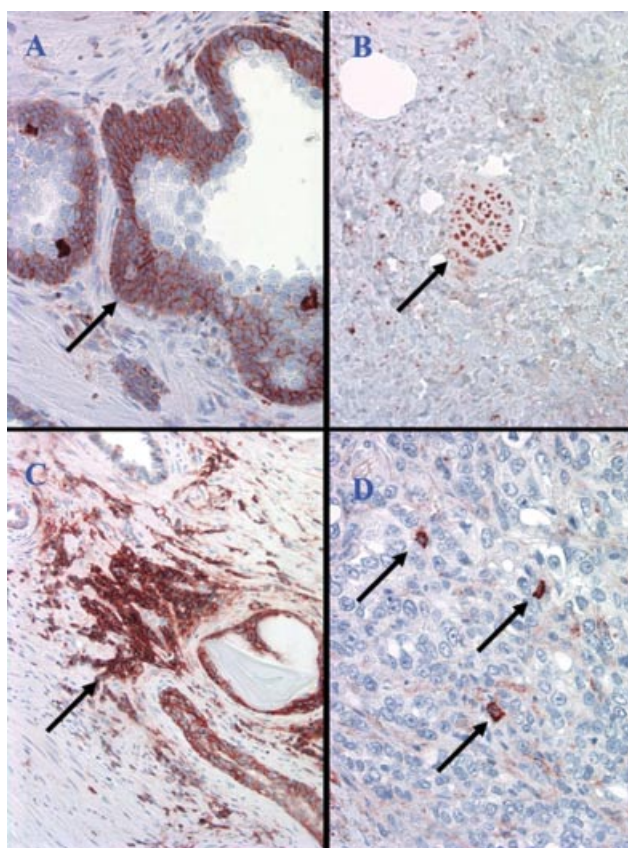
**Fig. 5.** Immunohistochemical study of the expression of CD44 in benign prostate and prostate cancer. In benign prostate, expression of CD44 is seen in (**A**) basal cells (arrow); (**B**) nerve (arrow); (**C**) lymphocytes (arrow). In prostate cancer (**D**), expression of CD44 is seen in scattered tumor cells, reminiscent of the distribution of neuroendocrine tumor cells (magnification 400×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

few exceptions, NE tumor cells were CD44 + cells; and CD44 + cells, minus a population of lymphocytes, were all NE tumor cells (Fig. 6B). A representative area of PC with lymphocytes (CD45 + /CD44 + /CgA−) and an NE cell (CgA + CD44 + /CD45−) is shown in Figure 6C.

## DISCUSSION

The mechanisms by which PC cells proliferate in an androgen-deprived environment remain unclear. Current hypotheses focus largely on altered AR signaling in tumor cells, including amplification of the AR gene, increased AR protein stability, AR hypersensitivity to low levels of androgen, AR mutation and activation of mutant AR by non-traditional ligands (reviewed by Scher and Sawyers [33]). An alternate theory that has gained significant attention recently involves CSCs. The hierarchical CSC model predicts that the putative PC stem cell, unlike the bulk tumor cells, is AR negative

and androgen-independent. As a result, PC stem cells may be resistant to hormone ablation and responsible for tumor recurrence. Although many different markers have been reported to identify CSCs in PC [10–16,34–36], the comprehensive study by Patrawala et al. [20] as well as those by others, have provided convincing evidence that the CD44 + subpopulation of cells may demarcate the PC stem/progenitor cells.

PCs are composed mostly of secretory type epithelial tumor cells with a small population of morphologically and functionally distinct NE cells. NE cells are increased in high grade and high stage tumors, particularly in hormonally treated and hormone-refractory tumors [25]. The levels of circulating chromogranin A, a product of the NE cells, are increased in men with PC in comparison to patients with benign conditions. Furthermore, serum chromogranin A levels correlate with the stage of disease and is an independent prognostic factor in men with hormone-refractory disease [25]. An important feature of NE cells is that they do not express AR [22–24]. Thus, they may be resistant to androgen ablation and contribute to tumor recurrence after hormonal therapy. Animal studies using xenograft and genetic PC models support this view. Huss et al. reported that in the CWR22 human PC xenograft model, castration induces tumor regression followed by recurrence (androgen-independent tumor outgrowth). Notably, these investigators observed an increase in the number and proliferative activity of tumor NE cells after castration, suggesting that NE cells may promote tumor survival and resurgence [37]. Genetic animal models of PC also contain NE cells varying from very low in Pten−/− tumors [38] to high in tumors of TRAMP [39] and Rb-p53-mice [40]. Similarly, recurrent tumors in Pten−/− tumors after castration have been shown to be composed of significantly more NE cells than pre-castrate primary tumors [38].

In the current study, we have for the first time demonstrated unequivocally that NE cells are the only CD44 + tumor cells (i.e., non-lymphocyte/CD45−) in human PC tissue. In addition, we have ascertained an association of CD44 expression with cells expressing NE markers in three well-established human PC cell lines. Patrawala et al. [20] have shown that the AR− DU145 and PC3 cell lines, but not the AR + LNCaP cell line, express CD44. Leiblich et al. [41] found that NE markers are expressed in DU145 and PC3 cells, but not in LNCaP cells. Our results are consistent with these reports and indicate that in human PC cell lines, expression of the stem/progenitor cell marker CD44 is associated with cells with NE features. Furthermore, we confirmed the expression of NE markers from CD44 + cells in single cell suspensions obtained from fresh human surgical samples and human PC tissues at both the RNA and protein levels.
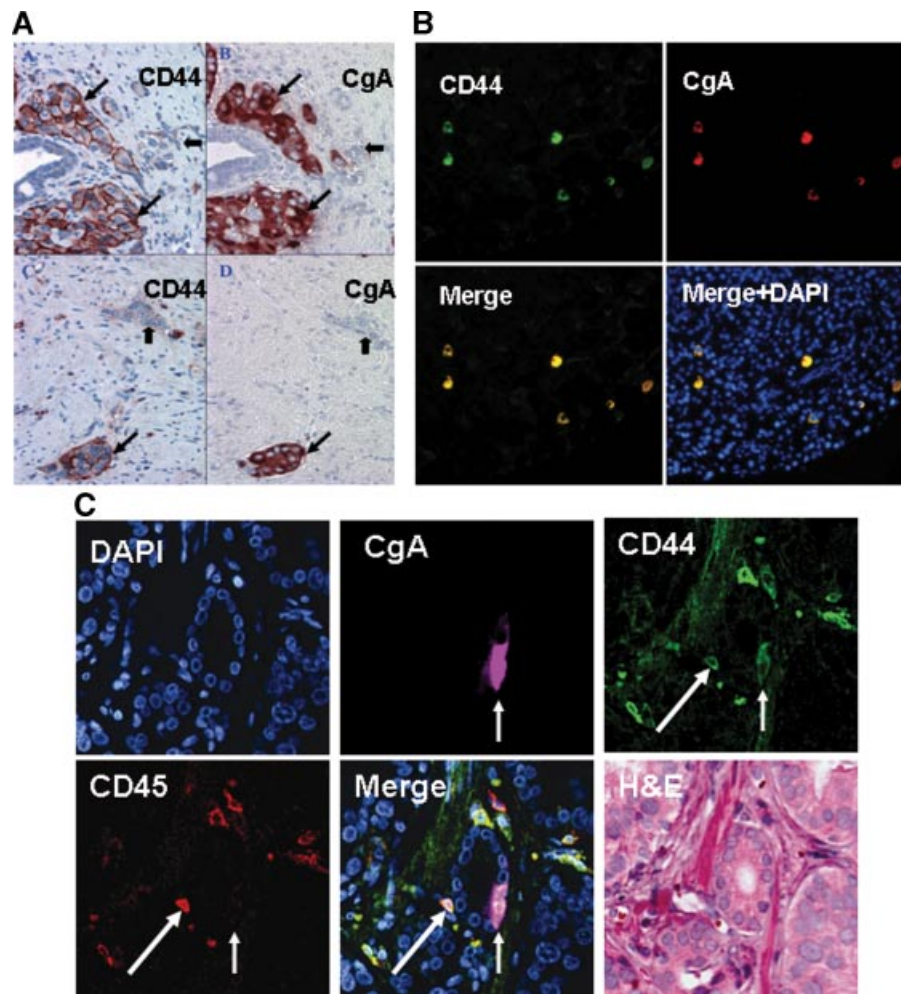
**Fig. 6.** Expression of CD44 is limited to NE tumor cells in human prostate cancer tissues. **A**: Immunohistochemical study of adjacent sections of a PC TMA for the expression of CD44 and CgA to show that NE tumor cells are CD44+ (long arrow) while non-NE tumor cells are CD44− (short arrow). **B**: A PC TMA slide was co-stained for the expression of CD44, CD45 and CgA by immunofluorescence study. In this field, there are no lymphocytes and all CD44+ cells are NE tumor cells (CgA+). **C**: In a different field, there is a single NE cell (CgA+) that is CD44+ and CD45− (short arrow). The other CD44+ cells are lymphocytes (CgA−, CD45+, long arrow) (magnification 400×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Using immunohistochemical and immunofluorescence studies of archival PC tissue in a tissue microarray, we showed that, excluding infiltrating lymphocytes (CD44 and CD45 double positive cells), expression of CD44, a putative CSC marker, is confined to NE tumor cells, an important observation that strengthens the hypothesis that NE cells within prostate tumors, being AR/PSA negative and normally quiescent [24,42], are possibly the therapy resistant cells responsible for tumor recurrence. These results are consistent with our recent finding that small cell carcinoma of the prostate, a tumor that is composed of pure malignant NE cells, consistently expresses CD44 [43].

**TABLE I. Expression of CD44, CgA, and CD45 in Cancer Areas of Human PC TMA (Total Nucleated Cells = 61,070)**

|  | CgA+ (NE cells) (n = 147) | CD44+ (NE cells + lymphocytes) (n = 648) | CD45+ (lymphocytes) (n = 516) |
|---|---|---|---|
| CgA− (n = 60,923) | — | 516 (79.6%) | 516 (100%) |
| CD44− (n = 60,407) | 15 (10.2%) | — | 0 (0%) |
| CD45− (n = 60,554) | 147 (100%) | 132 (20.4%) | — |

A recent publication shows that p53 inhibits expression of the CD44 to allow an untransformed cell to respond to stress-induced, p53-dependent cytostatic and apoptotic signals. In the absence of p53 function, the resulting CD44 expression is essential for the growth and tumor-initiating ability of highly tumorigenic mammary epithelial cells [44]. Significant expression of CD44 in NE tumor cells of PC suggests that these cells may be highly tumorigenic, as has been proposed for CSCs, challenging the concept that NE tumor cells are terminally differentiated, post-mitotic and play no role in cancer progression. This hypothesis is also consistent with the observation by Patrawala et al. [20] that the CD44 +, AR− PC cells can give rise to CD44−, AR + cells.

The reverse analysis showed that approximately 90% of the NE cells express CD44 while the remaining 10% were CD44−. Although this suggests the possibility of heterogeneity within the NE population, we cannot rule out false negative CD44 staining in some NE cells due to a sample bias based upon technical issues. For example, tumor cells in tissue section may not have been uniformly sectioned and hence focal membrane staining for CD44 may be missed in rare cells.

The origin of NE cells in the prostate remains controversial. NE cells are present in benign prostate as well as all stages of prostatic carcinogenesis, from PIN [45] to invasive carcinoma to metastatic PC [46,47]. It has been proposed that they may be derived from the same stem cell or pluripotent cell that gives rise to luminal secretory cells [48,49]. A population of proliferating/transit amplifying intermediate cells has been identified and postulated to be a common precursor for NE cells and other epithelial cells of the benign prostate [50,51]. The same has been assumed for the NE cells in PC which are considered to share the same stem/precursor cells with the secretory type cancer cells; although no definitive experimental evidence has been reported. Alternatively, some investigators favor the trans-differentiation model of NE cell origin, which suggests that the tumor NE cells are derived from the non-NE secretory-type tumor cells. For example, in in vitro assays, LNCaP cells, an androgen-dependent cell line, can be induced to show NE-like phenotype by androgen deprivation [52] or agents that increase intracellular levels of cAMP [53]. Our results, in combination with recent publications, would suggest an entirely different view, that is, at least in cancer, NE cells may themselves represent the stem/progenitor cells for the bulk differentiated, secretory type cancer cells. This may have profound implications on the treatment of PC as it suggests that only therapies that target NE cells, in combination with hormonal therapy that target the bulk tumor cells, would have the potential of curing men with lethal PC.

The CSC concept may have different meanings in different contexts. As summarized by Jordan et al. [5] CSCs can (i) be the source of all tumor cells in a primary tumor, (ii) comprise the small reservoir of therapy-resistant cells that are responsible for tumor recurrence after therapy-induced remission, and/or (iii) give rise to metastatic tumors. Because of the difficulty associated with purifying NE cells from fresh human PC tissue, functional studies on NE cells have not been reported. However, current evidence suggests that they may represent the hormonal therapy-resistant cells that are responsible for tumor recurrence; thus fulfilling a functional definition of a CSC. Based upon the present study, further functional and mechanistic studies are warranted to establish the role of NE cells as the putative PC stem cell.

## CONCLUSION

We have provided strong evidence that CD44, a marker that has been shown to be associated with increased tumorigenic potential in PC cell line and xenograft tumors, is expressed selectively in NE cells of human PC. This finding, in combination with the fact that such tumor cells do not express AR and are likely androgen-independent, further suggest their potential roles in tumor recurrence after hormonal therapy.

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